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PULSE VOLTAMMETRY IN SINGLE CELLS USING PLATINUM MICROELECTRODES

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ABSTRACT

Multiple pulse voltammetry at platinum microelectrodes is described for intracellular measurements. In this technique, a sequence of three potential pulses is used for each current point measured. This pulse sequence provides a fixed cathodic activation potential and a fixed anodic cleaning potential before a varying detection potential, at which the current is measured. Voltammetric information is obtained by ramping the detection potential stepwise through the potential range of interest. The multiple pulse voltammetry technique has been applied to the study of the oxidation of potassium ferrocyanide, glucose and several catechols at platinum microdisk electrodes. In addition, this technique has been applied at ultrasmall platinum ring electrodes to reduce electrode fouling during intracellular voltammetry. In these *in vivo* experiments, the electrode response is degraded by only 30% after 40 minutes of intracellular voltammetry.

INTRODUCTION

Many biologically important substances are electroactive, making them well-suited for electrochemical analysis. Voltammetry offers a number of advantages for the study of these compounds. These include a short analysis time and the ability to perform dynamic measurements in microenvironments. However, many biological samples contain very complex chemical matrices. These compounds and products from electrochemical oxidation, whether electrochemically active or not, often have a tendency to adsorb on the surface of voltammetric probes. Strong adsorption, for example that of high molecular weight species in the cell cytoplasm (proteins, lipids, sugars etc.), on the electrode surface usually results in a reduction of the voltammetric signal. Voltammetric signals have been found to deteriorate within minutes after implantation of the probes into biological samples [1,2]. Continuous detection of sample species, either qualitatively or quantitatively, becomes very difficult under these conditions.

Many aliphatic alcohols and amines are predicted to be easily oxidized based on thermodynamic data. However, they undergo oxidation at very slow reaction rates and are often considered not electroactive in the potential range used in aqueous solutions. Johnson and coworkers [3,4] have recently

pointed out that these slow reaction rates result from the absence of π-resonance by aliphatic free-radical products in the electrooxidation reaction. Lower activation energy can be achieved when partially unsaturated surface d-orbitals at noble metal electrodes (e.g. Pt and Au) overlap with these free-radical products and stabilize them. However, the same properties that lead to adsorption of these solutes on electrodes also lead to fouling of the electrodes by the accumulated reaction products.

Johnson and coworkers [3-12] have developed a pulsed amperometric detection technique for HPLC that minimizes electrode fouling while maintaining the reactivity of gold and platinum electrodes towards the aliphatic analytes. In this technique, a sequence of three potential pulses is used for each current point. After an oxidation detection potential pulse, a positive potential pulse oxidatively cleans the electrode surface, followed by a negative potential pulse which dissolves the passivating oxide layer formed during cleaning and restores the native reactivity of the clean metal surface. Successive electrochemical pretreatment steps prior to each current measurement allow continuous amperometric monitoring of many species ordinarily not determined electrochemically. Pulsed amperometric detection has been incorporated in liquid chromatography as a useful post-column detection technique for the determination of carbohydrates, amino acids and sulphur compounds [3,9,13,14,15]. A method involving pulsed activa-

tion of platinum or gold electrodes combined with stepping the detection potential to obtain voltammetric information for both qualitative and quantitative analysis has not been demonstrated to date.

In this communication, we describe the development of multiple pulse voltammetry at platinum microelectrodes. The theory of multiple pulse voltammetry has been described by Osteryoung and coworkers [16,17]. Our purpose in developing this pulsed voltammetry is to maintain the reactivity of the electrode surface in biological environments. The multiple pulse method described has been examined for several systems including the oxidation of potassium ferrocyanide, glucose, catechol and other neurologically important compounds at platinum microdisk electrodes. Furthermore, intracellular voltammetry at ultrasmall platinum ring electrodes has been carried out using the same technique to minimize the degree of electrode fouling *in vivo*.

EXPERIMENTAL SECTION

Reagents. Potassium ferrocyanide, glucose, dopamine (DA), dihydroxyphenylacetic acid (DOPAC), catechol (CAT) and ascorbic acid (AA) (Sigma Chemical Co., St. Louis, MO) were used as received. All buffers were prepared in doubly distilled water (Corning Mega-Pure MP-3A purification system) and were purged with nitrogen for 20 minutes before voltammetry. A blanket of

nitrogen was maintained over the solution for *in vitro* experiments to prevent air oxidation of dopamine. All experiments were carried out at room temperature.

Electrodes and Apparatus. Platinum disk working electrodes were fabricated from 10 and 75 μm diameter platinum wires (99.9%, Goodfellow, Cambridge, U.K.) sealed in soft glass tubes using a methane-oxygen torch. Electrical contact was made by connecting a stainless steel wire to the assembly with silver epoxy (Epo-Tek H20e, Epoxy Technology Inc., Billerica, Ma., cured at 110°C for 90 min). Electrodes were polished on a piece of abrasive grinding paper (320 grit, Buehler Ltd., Lake Bluff, Il.) followed by alumina powder (1 μm and 0.05 μm) before use. Final electrode structural diameters were approximately 2 mm providing a relatively shielded microelectrode. Ultrasmall platinum ring electrodes (1-5 μm structural tip diameters) were used for *in vivo* work. Platinum ring electrodes were constructed by placing carbon ring electrodes [18] in a solution of 1.0×10^{-3} M H_2PtCl_6 and 0.5 M H_2SO_4 and reducing platinum at 0.0 V vs SSCE for a desired deposition time. Cyclic voltammetry and pulse voltammetry were carried out with an EI-400 potentiostat (Ensman Instrumentation, Bloomington, IN). An IBM PC and a commercial interface (Labmaster, Scientific Solutions, Solon, OH) were used to generate potential waveforms for multiple pulse voltammetry and for data acquisition. A copper mesh Faraday cage was used to minimize interference noise. *In vitro* experiments were performed in a 30 mL

glass vial with holes drilled in a plastic cap to accommodate a two-electrode system. A three-electrode system was employed for *in vivo* experiments. A sodium saturated calomel electrode (SSCE) served as the reference electrode and a platinum wire as auxiliary electrode. Electrochemical reversibility was characterized by the "waveslope", which is the slope of a plot of potential (E) vs -log $\{(i_{lim}-i)/i\}$, to check for deviation from Nernstian behavior.

Intracellular Measurements. Preparations for intracellular voltammetry were similar to those described previously [19]. *Planorbis corneus* were obtained from NASCO (Fort Atkinson, WI) and were maintained in aquaria at room temperature until used. The snails were pinned in a wax-filled petri dish and dissected under snail Ringer's solution (39 Mm NaCl, 1.3 Mm KCl, 4.5 Mm $CaCl_2$, 1.5 Mm $MgCl_2$, 6.9 Mm $NaHCO_3$ Ph 7.4) [20] to reveal the left and right pedal ganglia. A micromanipulator (de Fonbrune, Curtin Matheson) was used to place an electrode into the identified dopamine neuron [21]. The electrode potential was monitored with an oscilloscope vs a platinum wire during implantation. A negative shift in potential was indicative of cell penetration. Following implantation, the platinum ring working electrode was connected to the potentiostat. The SSCE reference electrode was placed via a salt bridge into the Ringer's solution with a platinum wire serving as the auxiliary electrode. The experimental protocol involved 100 repeated multiple pulse voltammograms. Electrodes were calibrated both

prior to (pre) and after (post) the intracellular measurements.

RESULTS AND DISCUSSION

Description of the Method. Voltammetric techniques utilizing multiple pulse waveforms should provide the means to (i) obtain reproducible voltammograms in biological media over a long period of time, and (ii) obtain voltammetric information for solution species that are not typically considered easily oxidizable, including aliphatic amines, alcohols and other biologically important compounds. These oxidation reactions are facilitated with a multiple pulse amperometric scheme where pulsed potentials lead to reactant adsorption, oxidation and electrode cleaning [3,4]. A logical extension of this methodology is the use of a waveform scheme that involves multiple potential pulses to facilitate electrode cleaning and activation while linearly increasing the oxidation potential pulsewise to obtain current-potential curves.

The multiple pulse voltammetric technique described here employs a large anodic potential excursion (Ea) and a large cathodic potential (Ec), held for a duration of t_a and t_c, respectively, applied between each detection potential pulse (Ed) of duration t_d. A timing sequence for the multiple pulse waveform used is shown in Figure 1. The potential of Ed is stepped linearly within a selected reaction potential range to

obtain a voltammogram. Faradic current is obtained by sampling the current at the end of each E_d pulse. The faradaic current measured is then plotted against the potential of the detection pulse to generate a steady state normal pulse voltammogram. Appropriate values of E_s, E_c and the range for E_d in multiple pulse voltammetry can be chosen from examination of voltammograms obtained by cyclic voltammetry or linear sweep voltammetry [3,13]. As pointed out by Sinru et al. [14] the potential and time of each pulse has a direct effect on the nature of the voltammetry observed. The potential and time for each pulse applied in this report have been optimized by empirical observations to obtain the best results in terms of reproducibility and to minimize the disturbance to the biological system (e.g. a single cell).

Multiple Pulse Voltammetry In Vitro. The multiple pulse method has been employed to obtain voltammetry in several in vitro systems. A cyclic voltammogram and a multiple pulse voltammogram for the oxidation of 1.00×10^{-2} M $Fe(CN)_6^{4-}$ at a 75 µm platinum disk electrode are compared in Figure 2. A well defined sigmoidal wave with a limiting current of 175 Na is obtained with the multiple pulse voltammetry technique as expected. Since this experiment consists of a normal pulse voltammetry waveform with added cleaning and activation pulses, the limiting current for a diffusion controlled oxidation at a shielded disk-shaped electrode can be approximated by the following equation

$$i = \frac{nFAD^{1/2}C}{\pi^{1/2} t_d^{1/2}} + 4rnFDC$$

where n is the number of electrons transferred, F is the Faraday constant, A is the electrode area, D is the diffusion coefficient, C is the concentration, t_d is the step time, and r is the electrode radius [1]. For an electrode that is not fully shielded, the steady-state term in this equation is slightly higher [2]. For an electrode radius of 3.75×10^{-3} cm, a concentration of 1.0×10^{-5} mol/Ml, a step time of 0.2 s and assuming a diffusion coefficient of 6.0×10^{-6} cm^2/s the predicted limiting current is 220 Na. Sigmoidal voltammetry is expected even at the larger 75 μm electrodes, provided that the depletion layer created during oxidation and cleaning is allowed to relax before the subsequent detection pulse, and provided that the reaction is diffusion controlled.

The electrochemistry of glucose has been widely studied [22-30]. Oxidation of glucose has been reported to be a complex process involving parallel and/or consecutive potential and concentration-dependent reactions at the electrode [22,23,30]. In addition, gluconic acid, a passivating species, is known to be a product of glucose oxidation in the potential range from -0.60 V to +0.25 V. Build-up of this passivating species on the electrode surface can reduce the active surface area and inhibits the oxidation of glucose [22,23]. Voltammograms for the oxidation of glucose by cyclic voltammetry and by multiple pulse voltammetry are compared in Figure 3. Figure 3a shows the first and tenth cyclic voltam-

metric scans for the oxidation of 5.0×10^{-3} M glucose at a 10 μm platinum disk electrode. The electrode response degrades by 92% after ten consecutive scans. This appears to be the result of electrode passivation from glucose oxidation products. Figure 3b shows corresponding voltammograms obtained using multiple pulse voltammetry, which show little or no loss of electrode response. Voltammograms in both 3a and 3b are peak shaped, which is unexpected at microelectrodes used under conditions of steady-state diffusion. This voltammetry can be explained by formation of higher oxides at potentials more positive than -0.3 V and a resulting reduction of the catalytic oxidation of glucose [30]. Since this observation is so unusual when compared to diffusion-controlled steady state electrochemistry at microelectrodes, it should be pointed out that this loss of catalytic activity for the oxidation of glucose has been thoroughly documented at gold electrodes by Johnson and coworkers [30]. The calibration plot of anodic peak current at -0.36 V vs concentration for multiple pulse voltammetry of glucose is shown in Figure 4. Although the overall calibration plot is curved, the plot is nearly linear from 1.0 to 40 Mm and has a slope of 0.32 Na Mm^{-1} and a correlation coefficient of 0.997 over this range of concentration. This negative deviation from linearity is similar to results obtained in other laboratories at larger gold and platinum electrodes [23,30].

Multiple Pulse Voltammetry of Neurochemicals. Figure 5 shows multiple pulse voltammograms for the oxidation of DA, DOPAC, CAT and AA at a 10 μm platinum disk electrode. Well-defined sigmoidal voltammograms are obtained in each case. The calculated waveslope and $E_{¼}$ for each system are 111 mV/decade and 196 Mv vs SSCE for DA, 257 Mv/decade and 156 Mv for DOPAC, 118 Mv/decade and 232 Mv for CAT, 191 Mv/decade and 251 Mv for AA. The waveslopes obtained for these systems are greater (less Nernstian like) than those obtained at carbon fiber [2] and carbon ring [18] microelectrodes by cyclic voltammetry. The dependence of the multiple pulse voltammetry limiting current on concentration for each of the above substances has also been investigated over the range from $5.0x10^{-5}$ M to $1.0x10^{-3}$ M. The linear correlation coefficients are 0.998, 0.999, 0.995 and 0.999 for DA, DOPAC, CAT and AA, respectively.

Multiple Pulse Voltammetry in Single Cell Cytoplasm. Structurally small electrodes (< 5 μm) [18,31-37] have been constructed and applied to measurements of neurotransmitters in single nerve cell cytoplasm [19,31,32,35,36]. These measurements can provide unique information concerning neurotransmitter storage and dynamics in single cells. Carbon ring electrodes have been implanted into a large dopamine neuron of the pond snail *Planorbis corneus* and the voltammetric results obtained suggest that at least 98% of total stored of endogenous dopamine in *Planorbis corneus* is bound and not directly accessible to the cytoplasm [35,36]. However, adsorption of

high molecular weight species present in the cell cytoplasm can cause a deterioration of the voltammetric response of the electrode, making these *in vivo* experiments very difficult. Recently, a linear-average calibration method, which assumes a linear dependence between the degree of electrode fouling and the number of scans taken in the neuronal microenvironment, has been suggested as a calibration method for small electrodes used in the biological microenvironment of a single cell [19]. In the present work, the effectiveness of minimizing electrode fouling at ultrasmall platinum ring electrodes in intracellular voltammetry has been investigated using the multiple pulse waveform.

Intracellular multiple pulse voltammetry has been carried out by implantation of a platinum ring electrode into the cell body of the DA neuron of *Planorbis corneus*. Calibration plots obtained with multiple pulse voltammetry before and after intracellular voltammetry are shown in Figure 6. From these plots, the average ratio of two current values from pre- and post-calibrations, selected in the lower and higher concentration range, respectively, has been used to calculate the fraction of the electrode response remaining after the intracellular experiment. Data acquired at 20 μM DA concentration during post-calibration has been used to estimate the detection limit. An average loss of 30±12% (n = 9) in electrode response and an average detection limit of approximately 5±2 μM (concentration equivalent to twice the peak-to-peak noise)

is obtained for naked platinum ring electrodes having 2-6 μm tip diameters after multiple pulse voltammetry has been carried out in the giant DA neuron. When staircase voltammetry is used to obtain voltammograms in this cell, the average loss in response is 74±3% with a final detection limit of 12 μM for a carbon ring electrode [19]. Hence, multiple pulse voltammetry at platinum electrodes appears to be an excellent method to minimize electrode fouling during intracellular experiments.

CONCLUSIONS

The use of multiple pulse techniques to obtain voltammetric information in static microenvironments offers significant advantages to biological analysis. First, several carbohydrates and amino acids should be amenable to analysis by this technique, whereas these substances are usually difficult to determine by voltammetric means. Data has been presented here for the voltammetry of glucose. Second, electrode fouling *in vivo* can be minimized leading to more reliable quantitative analysis. Multiple pulse voltammetry has been shown to reduce the percentage of electrode fouling to only 30% after 40 min of continuous voltammetry inside a single nerve cell.

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UPCOMING RESEARCH

Multiple pulse voltammetry at platinum electrodes is described for intracellular analysis. This technique has been shown to provide repeatable voltammetry for biological samples, and to minimize deterioration of voltammetric signals at electrodes used in biological microenvironments.

FIGURE CAPTIONS

Figure 1 Schematic diagram illustrating the waveform used for multiple pulse voltammetry. E_a denotes an oxidative cleaning potential held for a time of t_a, E_c an electrode reactivation potential held for t_c, while the detection potential, E_d, is held for t_d before the current is sampled at the end of each detection pulse.

Figure 2 (a) Cyclic voltammogram for oxidation of 1.00×10^{-2}M $Fe(CN)_6^{4-}$ in 0.10 M KNO_3 as supporting electrolyte at a 75 μm platinum disk electrode. Scan rate = 50 Mv/s.

(b) Multiple pulse voltammogram for oxidation of $Fe(CN)_6^{4-}$, with conditions as described in (a). Parameters set for the waveform: E_a = +0.5 V, t_a = 150 ms, E_c = -0.1 V, t_c = 150 ms, and E_d is stepped from -0.1 V to 0.5 V at 9.5 Mv/step, t_d = 200 ms.

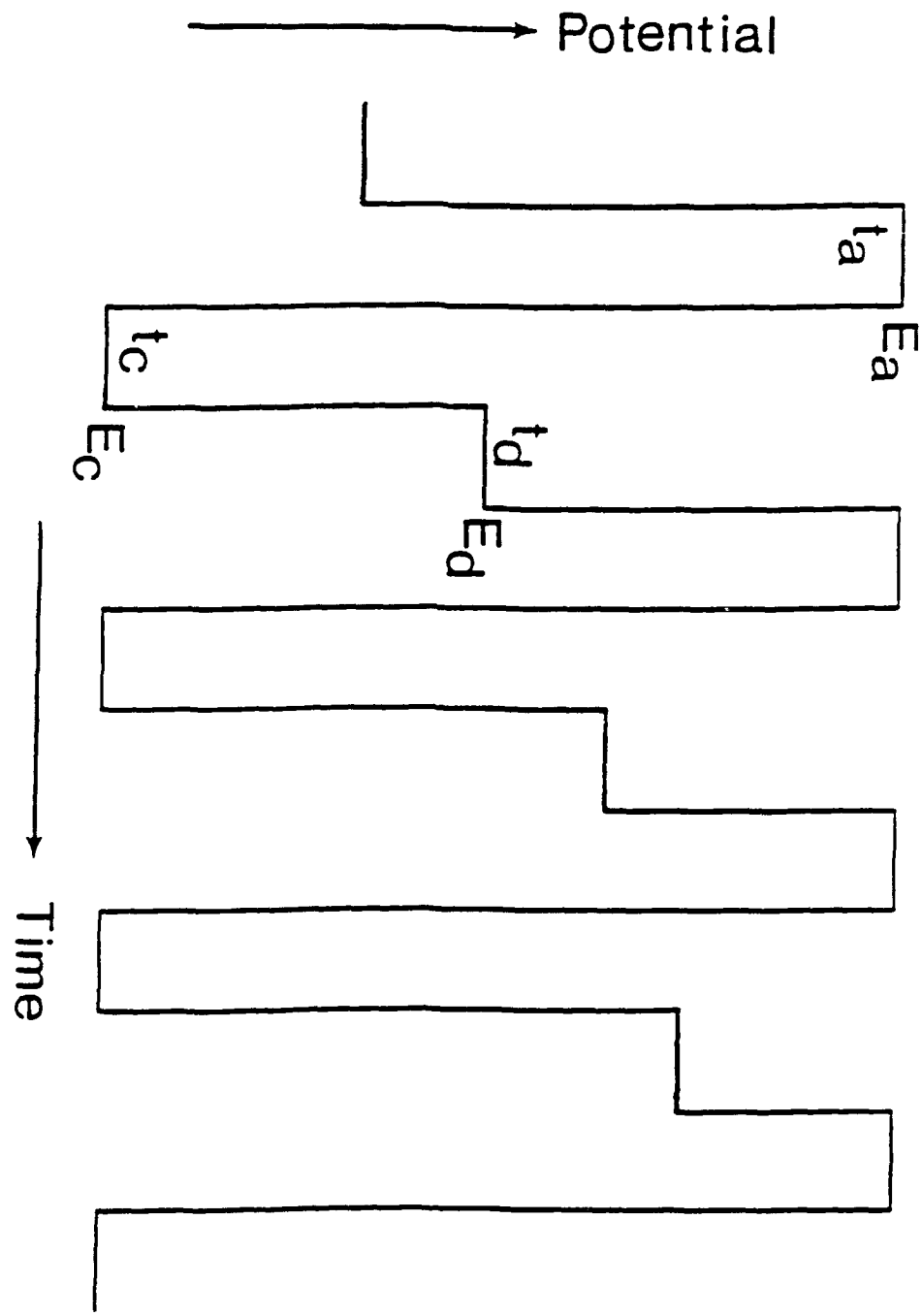
Figure 3 (a) Cyclic voltammograms for oxidation of 5.0×10^{-3}M glucose in Ph 7.4 citrate/phosphate buffer at a 10μm platinum disk electrode. Scan rate = 100 Mv/s. Numbers shown indicate the number of successive scans carried out.

Figure 3 (b) Multiple pulse voltammograms for oxidation of glucose under the same conditions as described in Figure 3(a). Parameters set for the waveform: E_a = +1.2 V, t_a = 120 ms, E_c = -0.6 V, t_c = 300 ms, E_d is stepped from -0.6 V to +0.1 V at 70 Mv/step, t_d = 150 ms. Squares and plus signs indicate the first and tenth successive runs carried out, respectively.

Figure 4 Plot of anodic peak current for multiple pulse voltammetry of glucose with a 10 μm platinum disk electrode at -0.36 V vs concentration of glucose. Current measured from a baseline recorded from blank buffer solution. Error bars indicate variations of the mean by one standard deviation (n=3).

Figure 5 Multiple pulse voltammetry for the oxidation of 7.4×10^{-4} M DA (a), DOPAC (b), CAT (c) and AA (d) in Ph 7.4 citrate/phosphate buffer at 10 μm platinum disk electrodes. Parameters set for the waveform: E_a = +0.8 V, t_a = 100 ms, E_c = -0.3 V, t = 40 ms, E_d is stepped from -0.2 V to + 0.8 V at 20 Mv/step, t_d =500 ms.

Figure 6 In vivo pre- and post-calibrations obtained in various concentrations of DA with a 2 μm tip diameter platinum ring electrode and multiple pulse voltammetry. The current shown in each data point represents the limiting current obtained from multiple pulse voltammograms. Parameters set for the waveform: E_a = + 1.0 V, E_c = - 0.3 V, E_d is stepped from -0.2 V to +0.8 V at 20 Mv/step, $t_a = t_c = t_d$ = 100 ms.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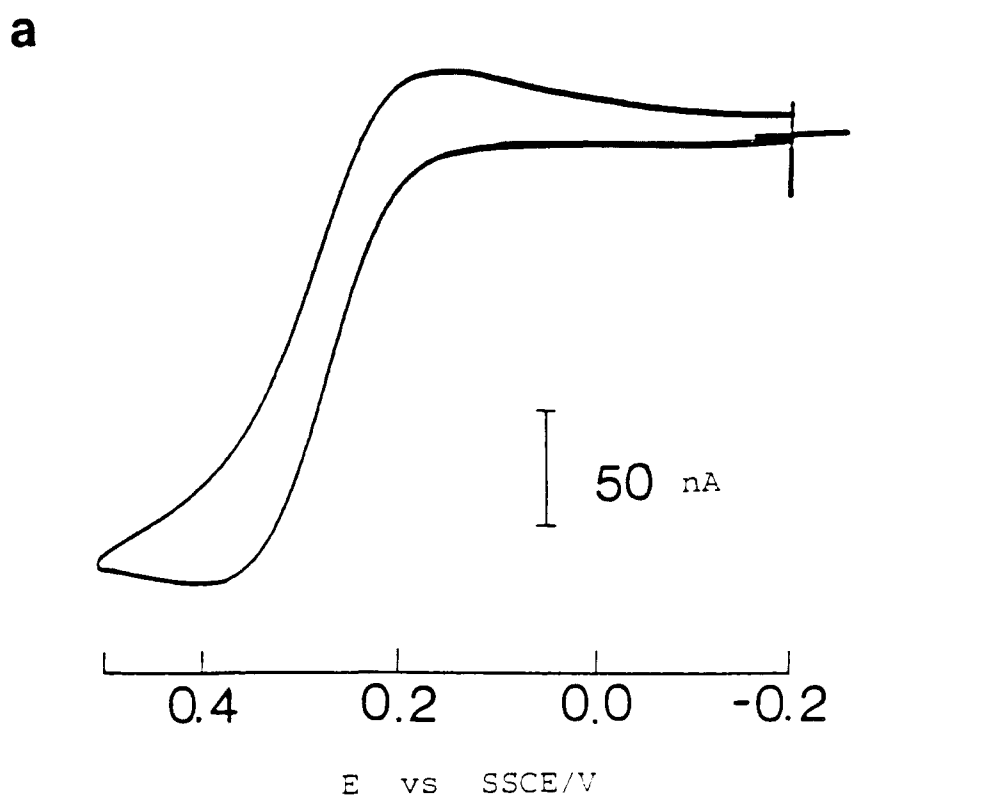
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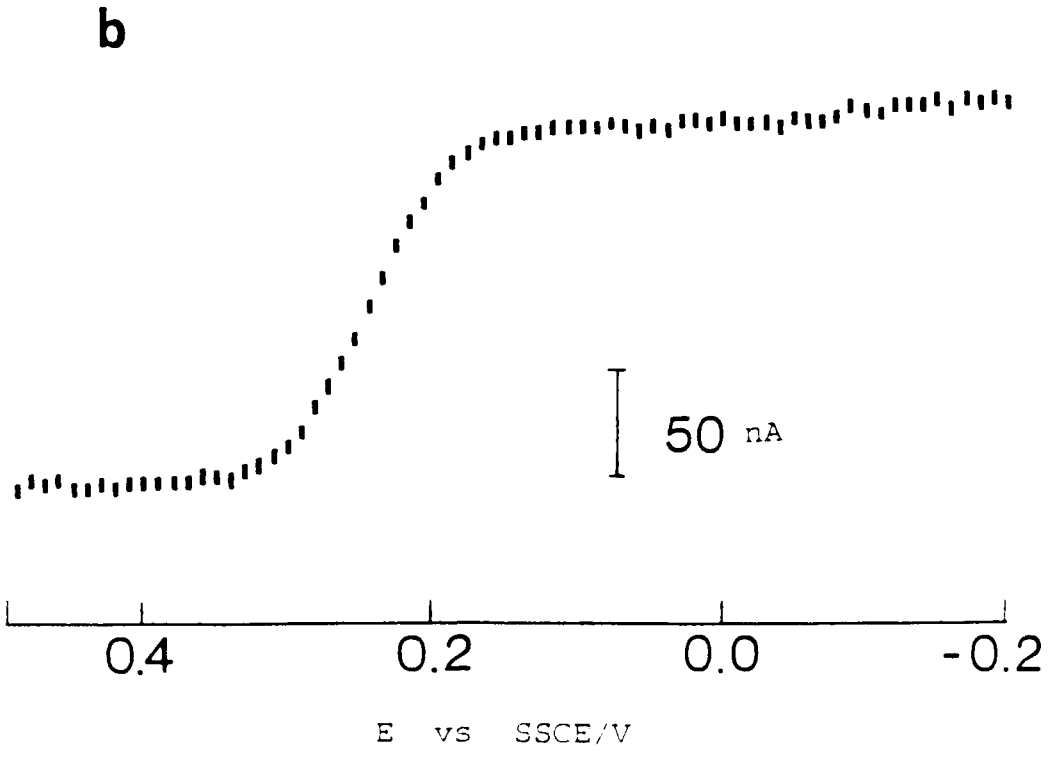
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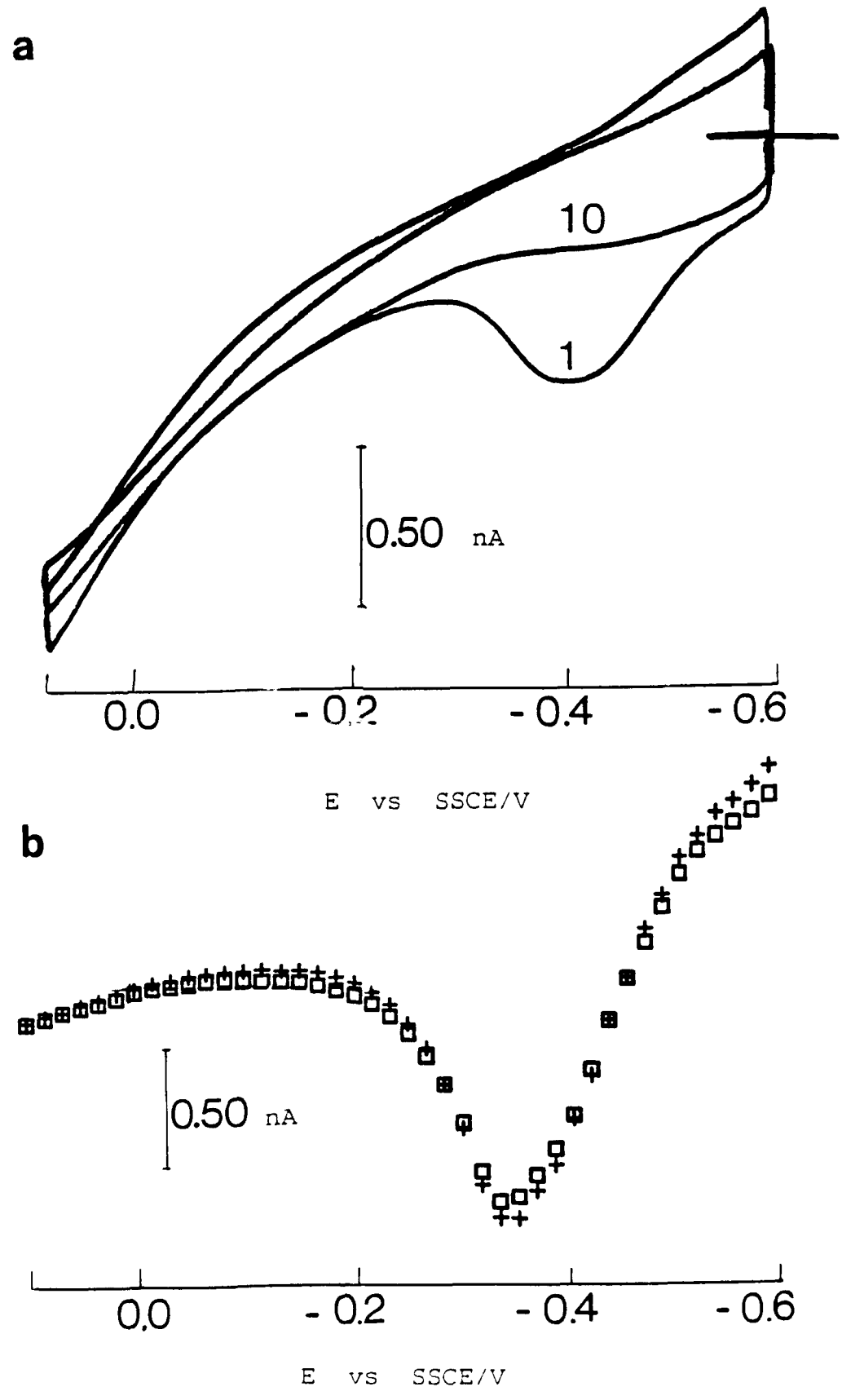
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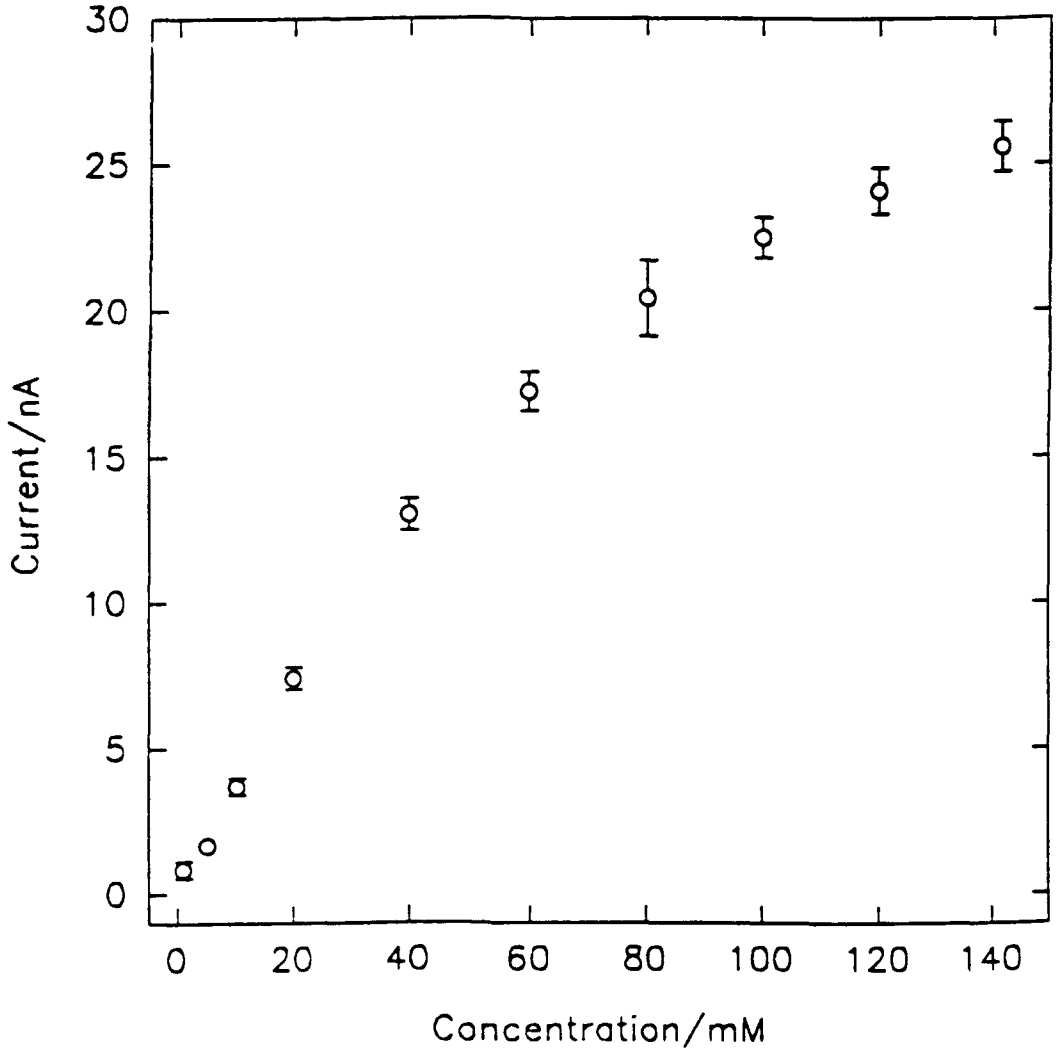
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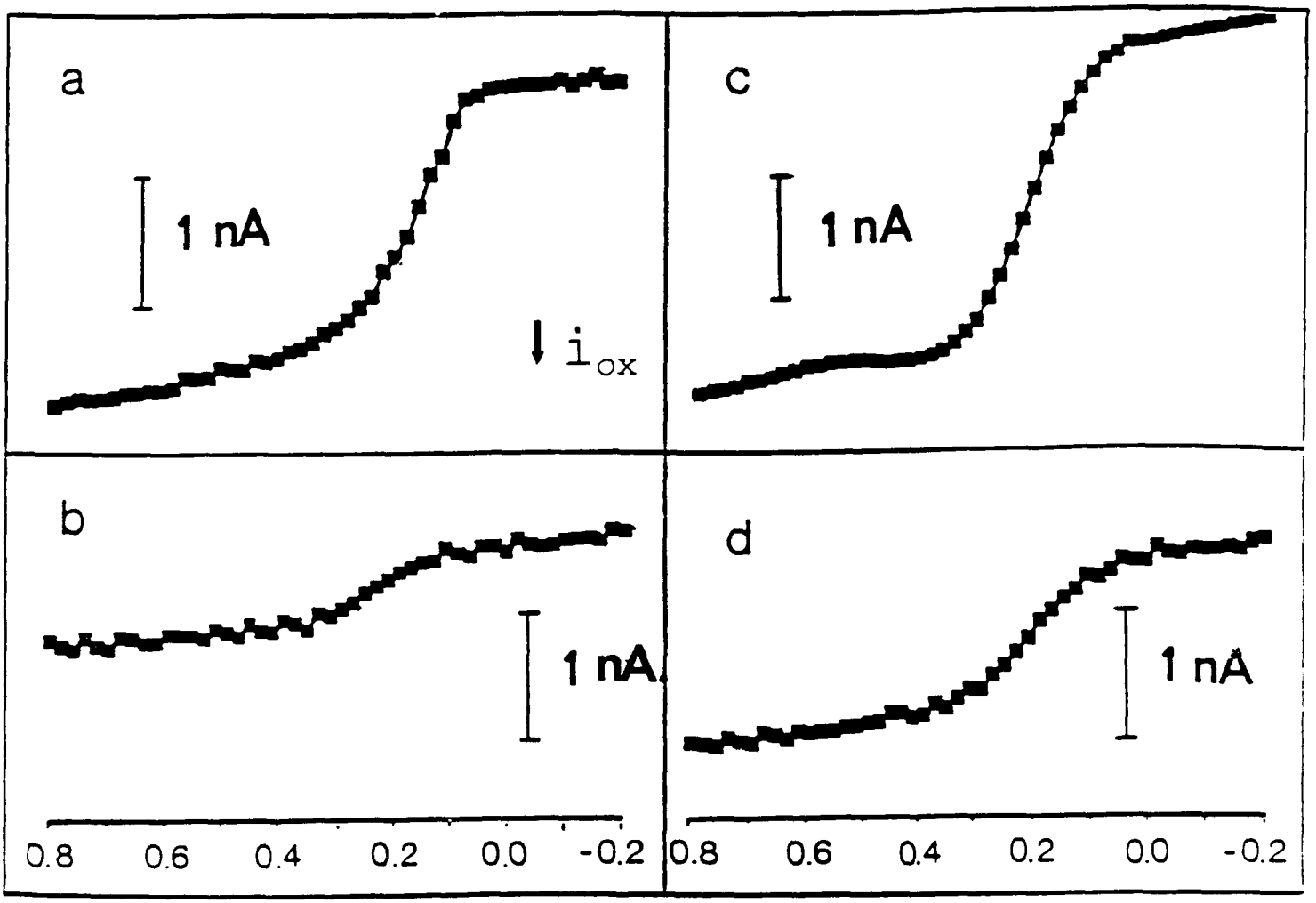
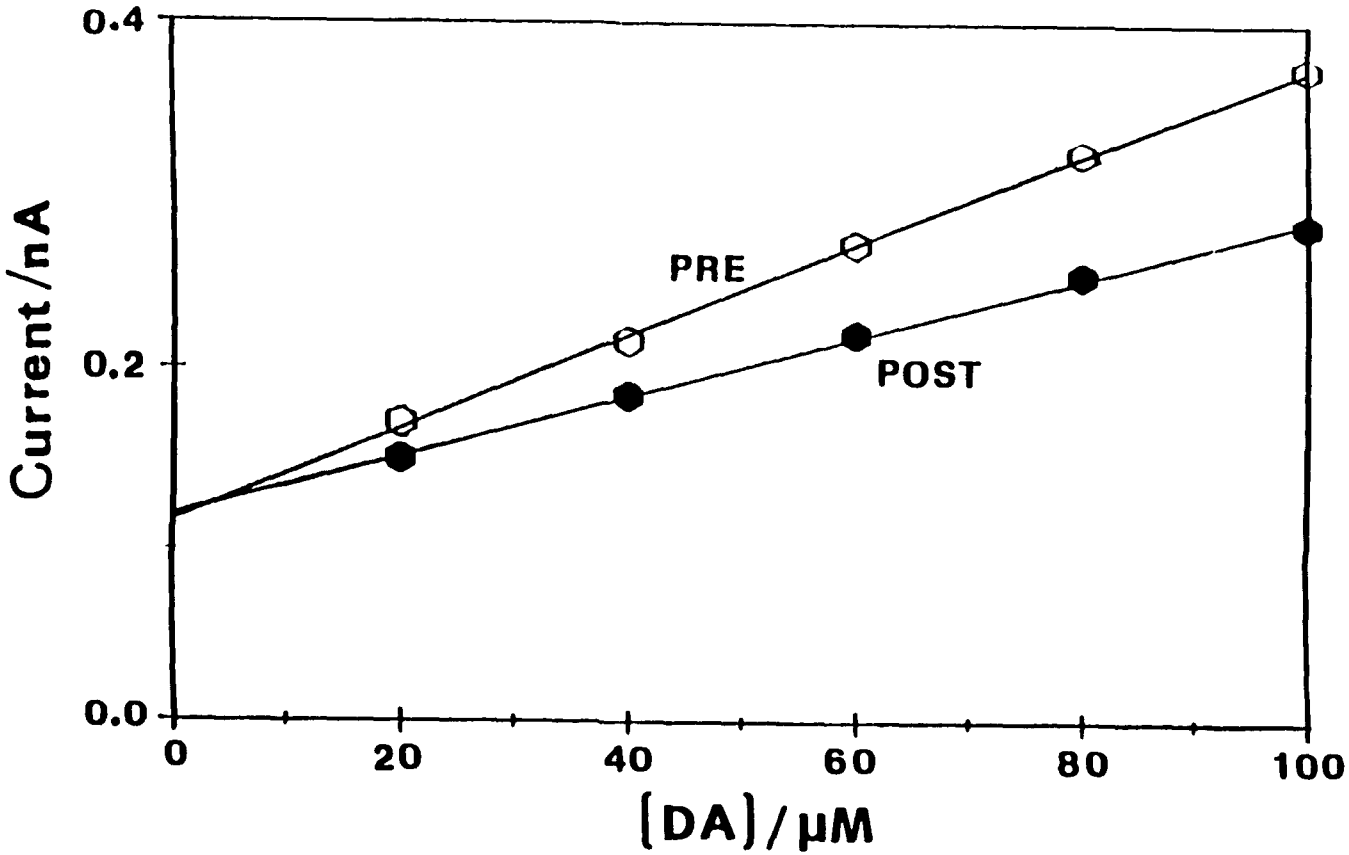


E vs SSCE / V


0.4
0.2
0.0
Current /nA
PRE
POST
0
20
40
60
80
100
[DA] / µM